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Original Article

Investigation on effects of the nourishing kidney and eliminating toxicity decoction on immunological imbalance of Th1, Th17 and Treg in HBV transgenic mice

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Abstract: Objective: To study the immune mechanism of nourishing kidney and eliminating toxicity decoction (NKETD) on Chronic Hepatitis B (CHB), we detected the serum concentrations of IFN-γ (the characteristic cytokine of Th1), IL-17A (the characteristic cytokine of Th17) and the quantitative proportion of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg in HBV transgenic mice. Methods: The HBV transgenic mice were randomly divided into six groups: high-dose group, middle-dose group, low-dose group, lamivudine group, model control group and normal mice control group. The serum concentrations of IFN-γ and IL-17A in mice were measured by ELISA method and the ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg was detected by Flow Cytometry Method (FCM). Results: The decoction could increase the serum concentration of IFN-γ and decrease that of IL-17A in HBV transgenic mice. The higher the dose was, the more significantly the concentration of IFN-γ increased. And high-dose decoction could decrease the serum concentration of IL-17A in HBV transgenic mice significantly and continuously while middle-dose and low-dose decoction had no significant effects. However, there wasn't statistically significant variation on the ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg in HBV transgenic mice. Conclusion: The decoction could treat CHB by regulating the immune function by promoting the generation of Th1 and/or enhancing its function while inhibiting Th17. The immune regulation by decoction had more significant effects than lamivudine.

Keywords: Nourishing kidney and eliminating toxicity decoction, Chronic Hepatitis B, IFN-γ, IL-17A, ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg

Introduction

Hepatitis B virus (HBV) infection is still a global health problem. About 400 million people worldwide are chronically infected with the virus, of whom 500,000 die each year from Chronic Hepatitis B (CHB) [1-3]. Interferon and nucleoside analogues (such as lamivudine) have a certain degree of efficacy in treating CHB. But the efficacy of these drugs is limited. For example, short term treatment of lamivudine can inhibit HBV replication by blocking the viral polymerase activity, while long term treatment would induce variations in the YMDD motif of the HBV polymerase and consequently lamivudine resistant strains were detected [4].

The key to eliminating HBV lies in specific cellular immunity [5]. It was suggested that HBV clearance required cellular and humoral immune responses, and non-HBV-specific immune response was possibly associated with liver damage and appeared to initiate the process of hepatic fibrosis [6]. T helper type 1 (Th1) lymphocytes played a pivotal role in virus clearance and liver damage by secreting interferon-gamma (IFN-γ) [7]. Recently, a novel and unique subset of IL-17A producing $CD4^+$ Th (Th17) cell highly enriched in liver of CHB patients has been described as a potential to exacerbate liver damage [8]. In addition, $CD4^+CD_{25}^+$ $foxp3^+$ regulatory T (Treg) cell appeared to restrain immune reactivity contributing to limit liver

damage [6]. In conclusion, accumulating evidence suggested that the imbalance of Th1, Th17 and Treg played an important role in leading to immune tolerance of HBV [6, 9].

The HBV transgenic mice selected in the experiment are mainly used for studying the immunological pathogenesis of HBV and evaluating therapeutic efficacy of anti-HBV drugs, which are a kind of relatively ideal animal model for evaluating and comparing anti-HVB drugs [10]. Traditional Chinese Medicine is not apt to increase the risk of drug resistance and its therapeutic efficacy is more stable [11]. The NKETD is one of the Traditional Chinese Medicines and a large number of clinical trials have proved its excellent therapeutic efficacy [11, 12].

In this study, we detected the serum concentrations of IFN-γ, IL-17A and the quantitative proportion of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg in HBV transgenic mice to analyze the immune mechanism of the NKETD in treating CHB, and consequently provided a certain theoretical significance for the clinical application of the NKETD.

Material and methods

Chemicals and reagents

170 g NKETD: parasitic loranthus: 20 g; semen cuscutae: 20 g; tu-chung: 10 g; phoenix-tail fern: 20 g; salvia miltiorrhizae: 20 g; gardenia fructus: 20 g; phyllanthus ruinaria: 30 g; radix fici hirtae: 20 g; pulp of cornus: 10 g; All the above medicinal materials were purchased from No. 1 Hospital Affiliated to Guangzhou University of Traditional Chinese Medicine and have been identified by pharmaceutical department.

Mouse IFN-γ Elisa Kit (Joyee Biological Technology Co., Ltd., Shanghai); Mouse IL-17A Elisa Kit (Joyee Biological Technology Co., Ltd., Shanghai); Phosphate Buffer Solution (Double Helix Biological Technology Co., Ltd., Shanghai); Mouse Peripheral Blood $CD^+_4CD^+_{25}$ foxp3 Treg Kit (eBioscience, US); Blood Cytolysate (BD Medical Instrument Co., Ltd., Shanghai); Lamivudine: 100 mg/tablet (Glaxo Smith Kline Limited); Normal saline: 250 ml: 2.25 g (Sichuan Kelun Pharmaceutical Co., Ltd., China). iMark Automatic Microplate Reader (Bio-RAD; US); FCM (BD Medical Instrument Co., Ltd., Shanghai).

Experimental animals

50 HBV transgenic mice; 10 normal mice (serum tests HBV-DNA negative). These were provided by Zhuangzhou Air Force Hospital Liver Disease Research Center.

Animal treatment

50 HBV transgenic mice were randomly divided into model control group, lamivudine group, high-dose decoction group, middle-dose decoction group and low-dose decoction group. Then take another 10 normal mice as normal control group.

Body surface area converting method combined with actual situation was applied for calculating feeding volume. The feeding volume was set as follows: male mice (> 25 g): 0.25 ml/day; female mice or male mice (< 25 g): 0.20 ml/day; concentrations of high-dose, middle-dose and low-dose groups were set respectively as 2 g, 1 g, 0.5 g/ml. Lamivudine group: 0.02 mg (lamivudine)/g (weight of mice). Model control group and normal control group: normal saline 0.2 ml/d or 0.25 ml/d.

Specimen collection

Collect the blood behind the eyeballs at 30th day and 60th day respectively. Get 200 μl whole blood and 100 μl whole blood respectively from each mouse. The 200 μl whole blood was for ELISA experiment. The 100 μl whole blood was for immediate FCM detection.

Concentration detection ofIFN-γ and IL-17A

Details of the techniques are provided in the Supplementary Methods I. In brief, add standard solutions (100 μl/hole) of different concentrations into corresponding holes, and add buffer solutions (50 μl/hole) and serum samples (50 μl/hole) into sample holes for sample analysis. After series of sample preparation, the OD_{450} value of these samples was immediately measured by ELIASA. The standard curve and its function formula can be evaluated through Microsoft Excel software.

The experimental preparations and detection procedures for IL-17A are the same as IFN-γ.

Detection procedure of the Ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg

Details of the techniques are provided in the Supplementary Methods II. In brief, Add CD_4

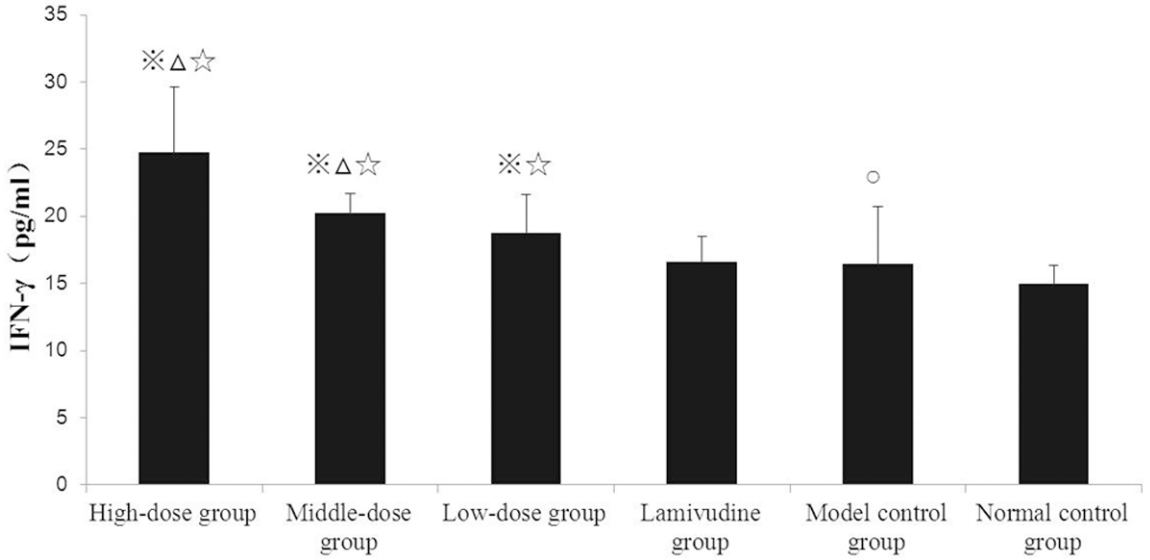

Figure 1. Comparison of Serum IFN-γ Concentration in Each Group at 30th day. Notes: 1. $^{※}P < 0.05$ compared to model control group; 2. $^{△}P < 0.05$ compared to low-dose group; 3. $^{○}P < 0.05$ compared to normal control group; 4. $^{☆}P < 0.05$compared to lamivudine group.

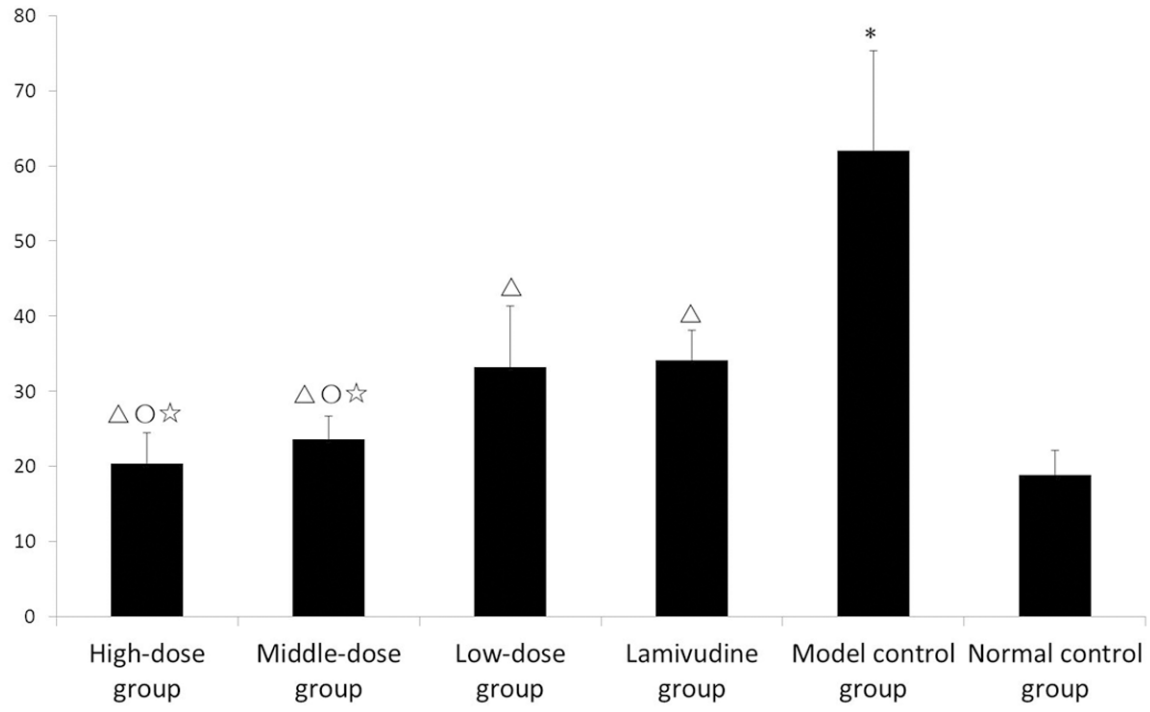

Figure 2. Comparison of Serum IL-17A Concentration in Each Group at 30th day ($\bar{x} \pm s$). Notes: 1. $^{*}P < 0.05$ compared to normal control group; 2. $^{△}P < 0.05$ compared to model control group; 3. $^{○}P < 0.05$ compared to low-dose group; 4. $^{☆}P < 0.05$ compared to lamivudine group.

antibodies (2 μl) and CD_{25} antibodies (2 μl) into flow-type tube, then add mouse anticoagulant whole blood (100 μl) respectively and let them react in a dark place at 4°C for half an hour. After series of sample preparation, these samples were immediately detected by FCM.

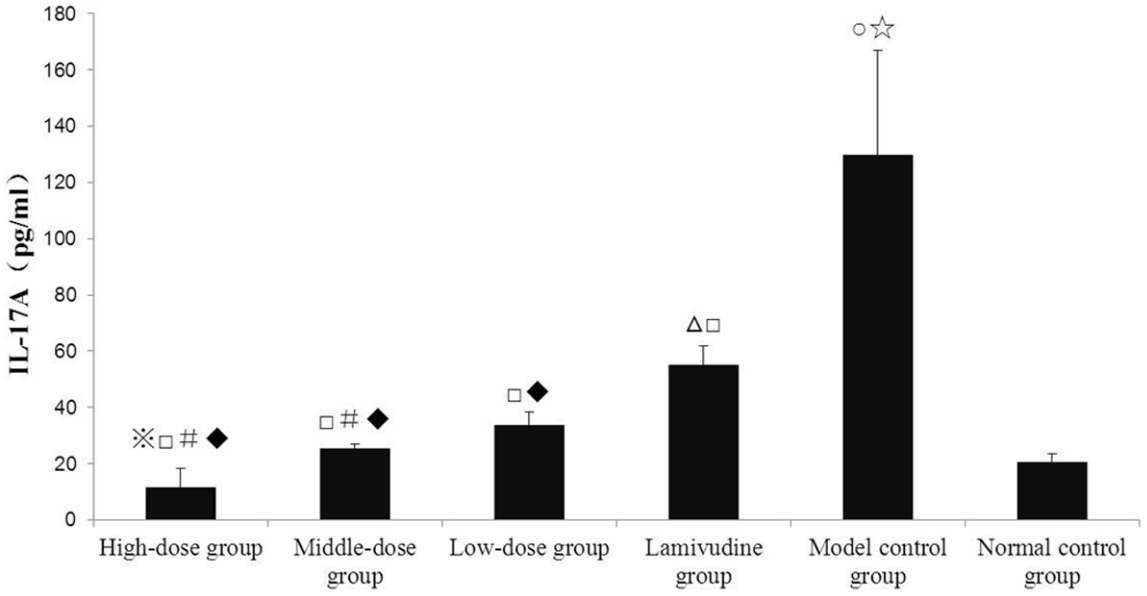

Figure 3. Comparison of serum IL-17A concentration in each group at 60th day was showed and serum IL-17A concentration in each group at 60th Day was compared to that at 30th day in **Figure 2**. Notes: 1. ※$P < 0.05$ compared to high-dose group in **Figure 2**; 2. △$P < 0.05$ compared to lamivudine group in **Figure 2**; 3. ○$P < 0.05$ compared to model control group in **Figure 2**; 4. ☆$P < 0.05$ compared to normal control group in **Figure 2**; 5. □$P < 0.05$ compared to model control group in **Figure 2**; 6. #$P < 0.05$ compared to low-dose group in **Figure 2**; 7. ◆$P < 0.05$ each level of decoction compared to lamivudine group in **Figure 3**.

Statistical analysis

All data were reported as mean ± standard deviation and analyzed using the Student's t test. The value of $P < 0.05$ was regarded as statistically significant. The data were analyzed statistically by SPSS 17.0 multivariate statistical analysis software.

Results and discussion

Treatment of NKETD decreased the serum IFN-γ concentration

The standard curve of IFN-γ standard solution is $y = 398.42x^2 + 153.11x - 9.3986$, $R^2 = 0.9994$. Since the serum IFN-γ concentration in the model control group increased significantly ($P < 0.05$) compared to that in normal control group, we suggested that HBV infection could induce the secretion of IFN-γ and consequently these cytokines could promote the immune system. After treatment of NKETD, the serum IFN-γ concentrations in high-dose, middle-dose and low-dose group all increased significantly ($P < 0.05$) compared to that in the model control group respectively. It was suggested that NKETD could further introduce the secretion of serum IFN-γ in HBV transgenic mice. All the serum IFN-γ concentrations in the three groups increased significantly ($P < 0.05$) compared to that in lamivudine group. However, the serum IFN-γ concentration in lamivudine group exhibited no significant variation compared to that in model control group, which suggested that NKETD had an advantage in treating CHB in the respect of immune function relative to lamivudine (classical anti-HBV drug). IFN-γ was the characteristic cytokine of Th1 [13], which suggested that NKETD could promote the generation of Th1 and/or enhance its function (**Figure 1**). Substantial evidence existed to support that Th1-type cytokines were selectively secreted during HBV infection, and Th1-mediated effects contributed to liver cell injury and eradication of HBV infection [7, 14, 15]. Our findings from this study further supported that Th1-mediated effects were highly relevant to HBV infection.

Treatment of NKETD decreased the serum IL-17A concentration

(1) At 30th day, the standard curve of IL-17A standard solution is $y = 73.818x^2 + 133.2x - 10.4$, $R^2 = 0.999$. The comparisons among groups at 30th day: The serum IL-17A concentra-

Table 1. The Ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg in Each Group at 60th day ($\bar{x}$)

Group	n	Ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg (%)
High-dose group	8	5.99
Middle-dose group	8	5.92
Low-dose group	9	5.57
Lamivudine group	7	4.88
Model control group	6	5.84
Normal control group	8	6.05

Notes: 1. n: the number of the mouse in the group.

tion in model control group increased significantly ($P < 0.05$) compared to that in normal control group. The serum IL-17A concentrations in high-dose, middle-dose, low-dose group and lamivudine group all decreased significantly ($P < 0.05$) compared to that in model control group respectively. The serum IL-17A concentrations in high-dose and middle-dose group decreased significantly ($P < 0.05$) compared to that in low-dose group respectively. The serum IL-17A concentrations in high-dose and middle-dose group decreased significantly ($P < 0.05$) compared to that in lamivudine group respectively (**Figure 2**).

(2) At 60th day, the standard curve of IL-17A standard solution is $y = 153.32x^2 + 114.35x - 8.7218$, $R^2 = 0.999$. The comparisons among groups at 60th day: The serum IL-17A concentration in model control group increased significantly ($P < 0.05$) compared to that in normal control group. The serum IL-17A concentrations in high-dose, middle-dose, low-dose group and lamivudine group all decreased significantly ($P < 0.05$) compared to that in model control group respectively. The serum IL-17A concentrations in high-dose and middle-dose decreased significantly ($P < 0.05$) compared to that in low-dose group respectively. The serum IL-17A concentrations in high-dose and middle-dose decreased significantly ($P < 0.05$) compared to that in lamivudine group respectively (**Figure 3**).

(3) Comparisons between 30th day and 60th day in the same group: The serum IL-17A concentration in high-dose group at 60th day decreased significantly ($P < 0.05$) compared to that at 30th day. The serum IL-17A concentration in lamivudine group at 60th day increased significantly ($P < 0.05$) compared to that at 30th day. The serum IL-17A concentration in model control group at 60th day increased significantly ($P < 0.05$) compared to that at 30th day (**Figure 3**).

By comparing model control group with normal control group, the serum IL-17A concentration in the former increased significantly, which suggested that HBV infection could significantly promote the secretion of IL-17A. Compared to the serum IL-17A concentration in model group, all these concentrations of groups significantly decreased to different degrees after the NKETD treatment or lamivudine treatment, which could be caused by immune regulation of NKETD or effect of lamivudine on HBV respectively. The higher the concentration of the NKETD was, the more significantly the serum IL-17A concentration decreased. Although the low-dose group exhibited no significant variation compared to lamivudine group, the serum IL-17A concentrations in high-dose and middle-dose group significantly decreased, which also suggested that the NKETD had an advantage in treating CHB in the respect of immune function relative to lamivudine. It was suggested that IL-17A was associated with neutrophil-mediated liver damage during HBV infection and IL-17A might exacerbate persistent liver inflammation and hepatic injury [6]. In addition, IL-17A was the characteristic cytokine of Th17 [8], which was increased in patients with hepatitis B and contributed to further deterioration of disease progression [16]. Our observation confirmed the interesting interaction between IL-17A and HBV infection. However, the detailed immune mechanism during HBV infection remained to be elucidated.

Change of Ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg was not statistically significant

The results of FCM were performed in Supplementary Figures 1-6. The ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg in model control group was not statistically significant ($P > 0.05$) compared to that in normal control group, but it tended to decreased. And the ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg in high-dose and middle-dose group increased slightly compared to that in model control group, however, it was not statistically significant ($P > 0.05$) (**Table 1**). $CD4^+$ T lymphocytes served a critical role in immune regulation, which was regarded as a highly heterogeneous population of cells characterized by the ability to secrete various types of cytokines and to assist other immune

cells [17]. However, it was suggested that a particular process like tissue damage could not be explained by single cytokine, because there were complex pathophysiological cytokine networks in tissue lesions [6, 18], and NKETD was reported to be associated with Th1 and Th17, which indicated that the immune mechanism of NKETD in treating CHB was worthy of further investigation.

Traditional Chinese Medicine is regarded as maintenance therapy and has increasingly become popular in the west, which has been explored widely [19]. Our findings from this study have confirmed that Traditional Chinese Medicine can exhibit more effective than some interferon and nucleoside analogues in some respects.

Conclusion

The NKETD could regulate the immune function in treating CHB probably by promoting the generation of Th1 and/or enhancing its function while inhibiting the function of Th17. It had a more significant effect than lamivudine on immune regulation.

Supplementary methods

Supplementary methods I: concentration detection of IFN-γ and IL-17A: Prepare IFN-γ standard solution and dissolve it into distilled water: 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml and 62.5 pg/ml. The concentrations of IL-17A standard: 62.5 pg/ml, 31.25 pg/ml, 15.63 pg/ml, 7.813 pg/ml and 3.906 pg/ml. Add standard solutions (100 μl/hole) of different concentrations into corresponding holes, add buffer solutions (50 μl/hole) for sample analysis into sample holes, add serum samples (50 μl/hole) into each sample hole, and then incubate them at room temperature of 25 to 28°C for two hours. After that, add diluted cleaning solutions (300 μl/hole), keep at least 15-second standing and pour the solution inside out. Clean the troughs 5 times. Put the plate on the thick absorbent paper and pat dry after rinsing the plate for the last time. Then add biotinylated antibody treatment fluid (100 μl/hole), seal the holes with gummed paper and incubate at room temperature for an hour. Add diluted cleaning solutions (300 μl/hole), keep at least 15-second standing and pour the solution inside out. Clean the troughs 5 times. Put the plate on the thick absorbent paper and pat dry after rinsing the plate for the last time. Then add enzyme bonder treatment fluid (100 μl/hole), seal the holes with gummed paper and incubate at room temperature for an hour. Add diluted cleaning solutions (300 μl/hole) in, keep at least 15-second standing and pour the solution inside out. Clean the troughs 5 times. Put the plate on the thick absorbent paper and pat dry after rinsing the plate for the last time. Then add TMB chromogenic agent (100 μl/hole) and incubate in a dark place at room temperature for 20 minutes. Then add stop buffer (50 μl/hole) and immediately put the plate into the ELIASA to measure OD_{450} value. The standard curve and its function formula can be evaluated through Microsoft Excel software and then their concentrations can be calculated respectively according to the standard curve.

The experimental preparations and detection procedures for IL-17A and IFN-γ are the same.

Supplementary methods II: detection procedure of the ratio of $CD^+_4CD^+_{25}$ foxp3 Treg to CD^+_4 Treg: Add CD_4 antibodies (2 μl) and CD_{25} antibodies (2 μl) into flow-type tube, then add mouse anticoagulant whole blood (100 μl) respectively and let them react in a dark place at 4°C for half an hour. Later, add diluted erythrocyte lysate (1 ml) (dissolved in ultrapure water, the volume ratio of it to ultrapure water is 1:5) and let them react for 5 minutes. Centrifuge them after adding PBS buffer solution (2 ml) (1500 rpm/min, 5 min/time, centrifugal temperature: 20°C, similarly hereinafter). After centrifugation, pour most of solution inside the flow-type tube out, add PBS buffer solution (2 ml) and re-centrifuge. Then pour the solution out, add intraprep permeabilization reagent (1 ml) (v/v (eBioscience Fixation/Permeabilization Concentrate: eBioscience Fixation/Permeabilization Diluent) = 1:3) and let them react for 6 hours. Add staining buffer (2 ml) (eBioscience Permeabilization Buffer dissolved in ultrapure water, v:v = 1:9) and then centrifuge. After centrifugation, pour the solution inside the flow-type tube out, add PBS buffer solution (2 ml), and then re-centrifuge. Pour the solution out, add Anti-Mouse CD16/32 (1 μl/tube) and let them react in a dark place at 4°C for 15 minutes. Then add foxp3 antibody (2 μl/tube) and let them react in a dark place at 4°C for 30 minutes. Add staining buffer (2 ml)

and centrifuge. After centrifugation, pour the solution inside the flow-type tube out, add PBS buffer solution (2 ml) and re-centrifuge. Then, pour the solution out, add staining buffer (400 μl/tube), and use FCM to detect.

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Disclosure of conflict of interest

None.

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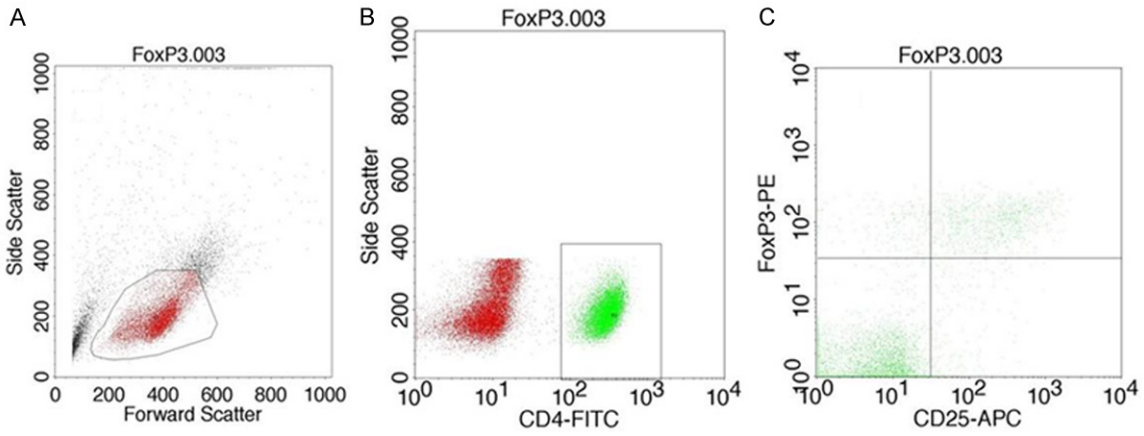
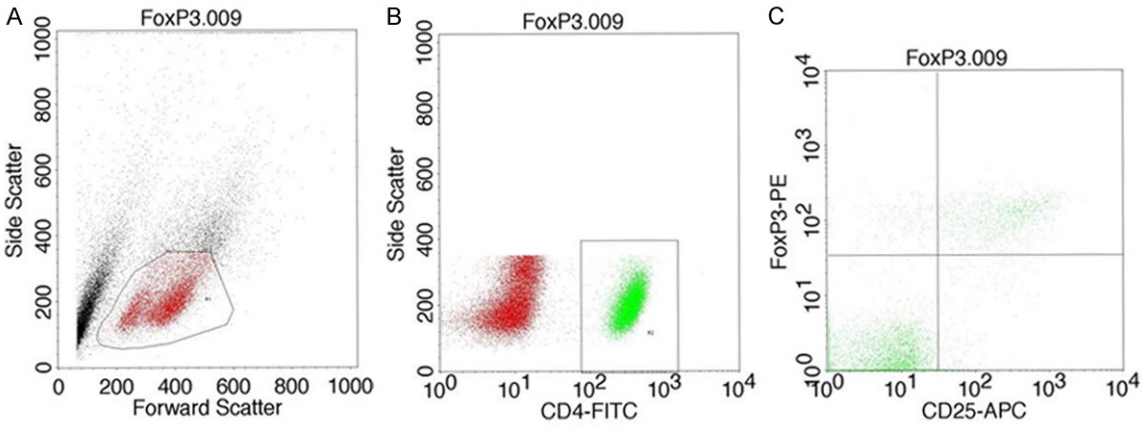
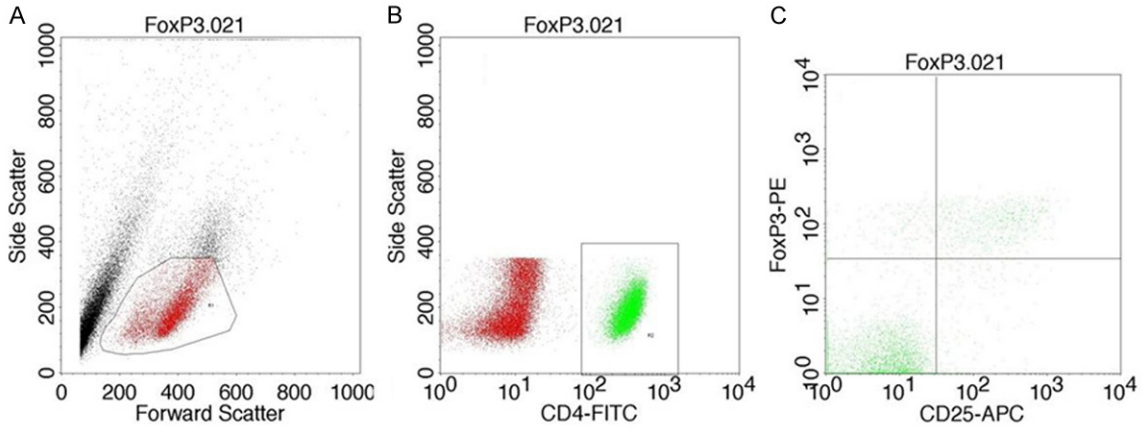


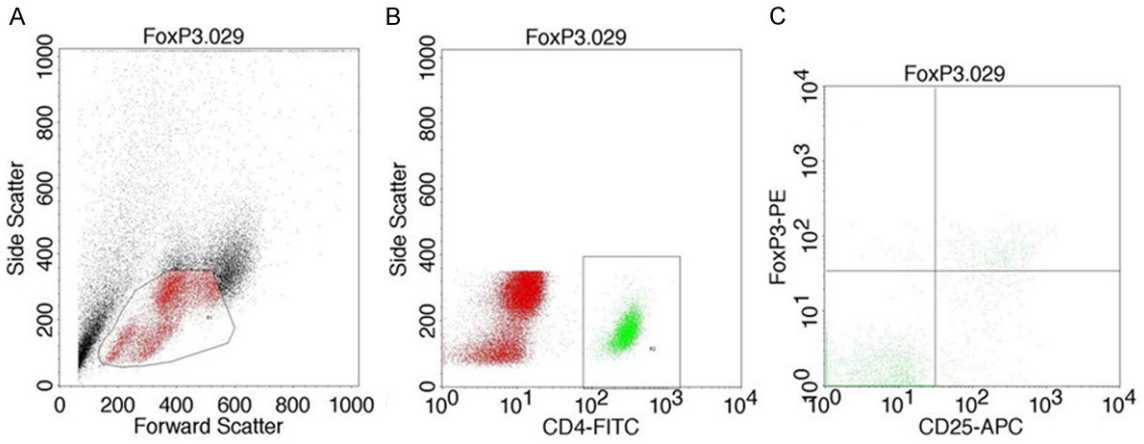
Supplementary Figure 1. Diagram of high-dose nourishing kidney and eliminating toxicity decoction.



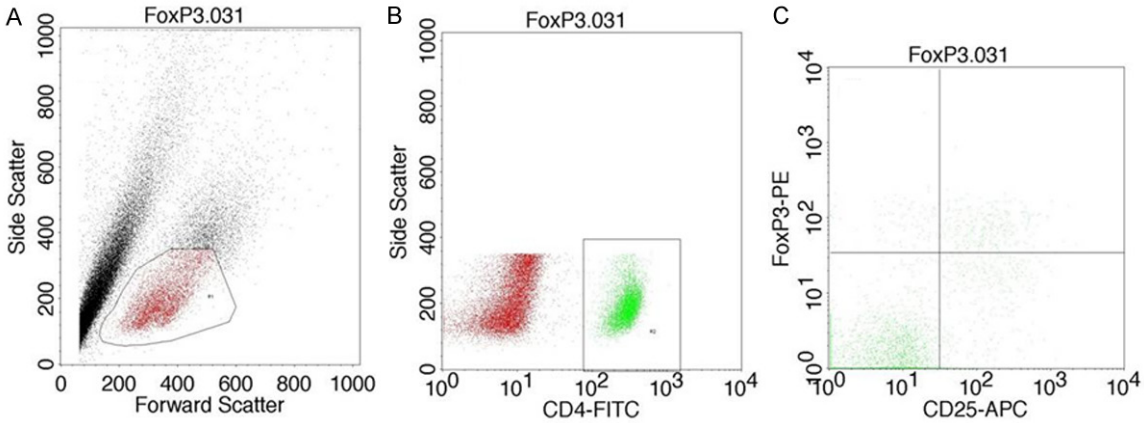
Supplementary Figure 2. Diagram of middle-dose nourishing kidney and eliminating toxicity decoction.



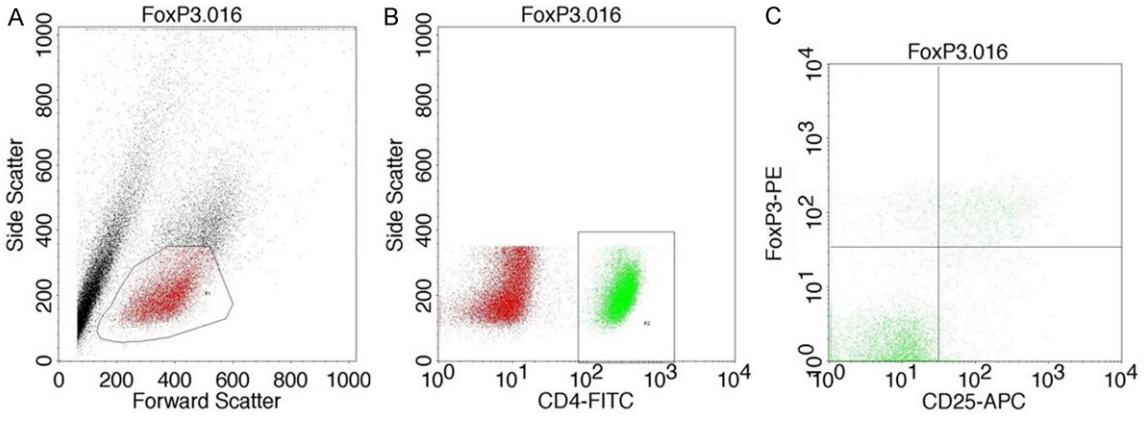
Supplementary Figure 3. Diagram of low-dose nourishing kidney and eliminating toxicity decoction.



Supplementary Figure 4. Diagram of lamivudine group.



Supplementary Figure 5. Diagram of model control group.



Supplementary Figure 6. Diagram of normal control group.